

# Issues in Biochemical Applications to Risk Assessment: How Do We Evaluate Individual Components of Multistage Models?

by Marshall W. Anderson\*

## Introduction

Let me give a few introductory remarks and see if I can stir up some questions. Figure 1 is a schema of the multistep process of transformation of cells by chemicals. There have been several versions of this during this symposium, and this is mine. There are essentially two issues that I want to raise about the multistep pro-

cess. Both of them will be a carryover of the last two discussions we've had.

The first issue is the so-called first step of transformation, which is the formation of an initiated cell. The formation of an initiated cell is in itself at least a two-step process, and there are many other factors involved. It involves the damage of the DNA either directly or indirectly by the agent. This damage is referred to as promutagenic lesions. In order to obtain an initiated cell, the damage must be fixed by replication.

The controversy over the formation of initiated cells is not in the formation of DNA damage but in this fixation step. How can the fixation occur? To bring this into focus with Ray Tennant's discussion (1), when testing mutagenic chemicals with the *in vitro* test or the Ames test, you obviously do not take the fixation step into account, because the cells of the bacteria are dividing. So if DNA is damaged, mutations are more than likely to occur.

However, in the whole animal the fixation step itself could be the limiting step. Jim Swenberg was kind enough to lend me a few slides and I want to illustrate with formaldehyde that you must take this step into account (2).

The tumor-response curve obtained with formaldehyde is very nonlinear. I will avoid using the word "threshold," but the slope here is probably approaching zero as the dose decreases. If you look at the promutagenic damage, i.e., the DNA adducts, the DNA adduct levels are fairly linear as you go to lower doses. Assume for purposes of argument that it is linear. Forget that it's formaldehyde. Thus, apparently, the formation of the promutagenic damage alone is not enough. But then, as Jim Swenberg and his co-workers have shown, the induction of cell turnover by formaldehyde is very dose dependent. In fact, the breaks in the tumor curve and in the curve showing the induction of cell turnover are similar. So at lower doses where you saw no tumor response, there was essentially no detectable induction of cell turnover.

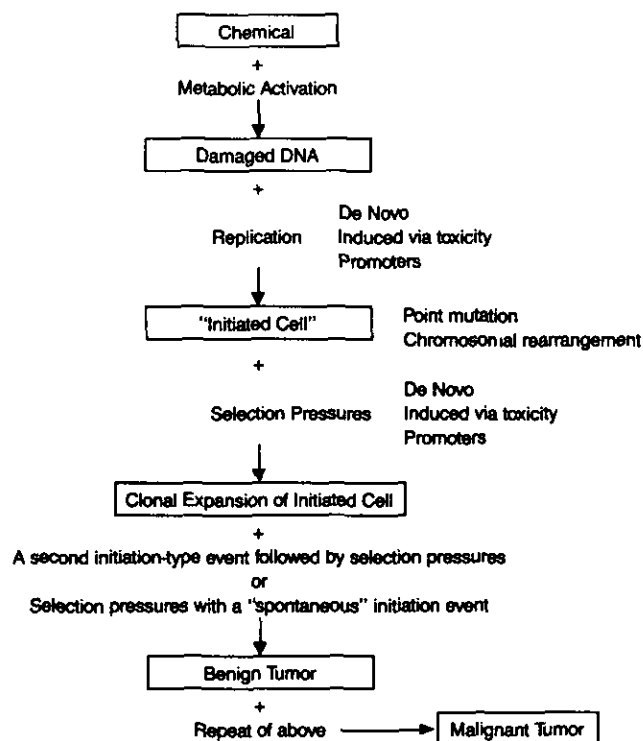


FIGURE 1. Multistep transformation of cells by chemicals.

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Again, I should say Jim Swenberg has pioneered the approach to examine cell replication in chronic administration of chemicals with carcinogenic regimens. Obviously, from this set of data (and I think there are similar examples) you would conclude that promutagenic lesions exist, but tumors are not evident because the damage is not fixed.

This can even occur with one chemical in the same animal, for example, with the tobacco specific nitrosamine 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (3). There are three tumor induction sites with NNK in the Fischer rat, and they have very different dose responses. The induction of nasal cavity and liver tumors probably requires the induction of cell turnover by NNK, but apparently that is not the case in the lungs.

Suppose a compound that is mutagenic in the Ames tests and other short-term tests has a linear response as far as promutagenic lesions are concerned, yet the slope of the tumor-response curve is decreasing. This could be a result of the lack of induction of cell turnover at lower doses. The question is how to regulate the compound. Should you regulate it based on the tumor response or based on the promutagenic lesions?

Obviously, there are pros and cons to either strategy. Personally, I don't want to be walking around with promutagenic lesions. Several presentations during Monday's session of DNA adducts reaffirm my conviction, based on two points. Firstly, some types of bulky DNA adducts are very persistent *in vivo*. For example, Miriam Poirier showed that a cisplatin adduct in humans was present 22 months after the last therapeutic dose (4). There are several examples of this. So people could be walking around with these adducts and they might get sick, which causes cell turnover. Thus, mutations would result. Secondly, Phil Hanawalt's data suggests that a given cell type only repairs the DNA damage in the active genes of that cell (5). The transcriptionally active genes are repaired, whereas the inactive ones are not. What happens if a chemical binds to an oncogene, for example, *c-mos*, that is inactive in most cells? If you induce a mutation in the promoter region of the gene itself, you could transform the cell. These possibilities require consideration.

There is another argument concerning the promutagenic lesions. An increasing amount of data shows that DNA damage could enter this process in several places. For example, the transformation of a benign tumor to a malignant tumor could result from a second-hit type phenomenon in the benign tumor. This is shown to be the case in the skin and liver systems, where second hit can increase the transformation from a benign tumor to a malignant tumor (6,7). Also, Julian Peto's data yesterday showed that older people exposed to radiation were more sensitive to tumor induction than younger people (unpublished observations). Thus, there is ample data to suggest that genotoxic lesions are also involved in the latter stages of malignancy.

The second issue I wanted to address about the multistep process concerns compounds that do not act by

genotoxic mechanisms and are promoters but not cytotoxic promoters. I agree that if a compound induces tumors only by cytotoxicity at high doses, then the chemical is probably safe. The arguable point is how to pick out chemicals (e.g., 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA); 2,3,7,8-tetra-chlorodibenzo-*p*-dioxin (TCDD); and hormonal promoters such as estrogen) whose mode of action is through specific receptors. How can we protect ourselves against compounds like TCDD? TCDD was completely negative in all genotoxic tests. I think at the doses administered in the bioassay studies, TCDD was not overly cytotoxic. So how can we identify this type of compound as having carcinogenic properties? One possible approach was outlined by Steve Reynolds in an earlier presentation at this conference (8). Comparisons of activated oncogenes between spontaneously occurring tumors and chemically induced tumors have the potential to identify non-genotoxic, noncytotoxic chemicals. Steve outlined this approach for the B6C3F1 mouse liver model. However, the approach can obviously be utilized in other animal model systems.

The following is a brief description of this approach. Assume the incidence of mouse liver tumors increased from 30% in control to 70% in treated animals. Analysis showed that the pattern of activated oncogenes were the same in treated and controls. In addition, a careful analysis of cell turnover was done at the doses employed in the carcinogenesis study. If there was chemically induced cell turnover at the doses employed, one could argue that the chemical is just cytotoxically promoting these spontaneous lesions. But suppose there was no cytotoxicity, as with TCDD? In this case, the chemical may be doing something very specific, like acting through a receptor. We need to take advantage of some of these sensitive *in vivo* model systems to analyze individual steps in the carcinogenesis process.

## Discussion

DR. RAYMOND TENNANT, NIEHS: In terms of the identification of tumor promoters, I think this is one excellent way, involving the mouse, at least. As a point of departure, I would really like to offer the possibility that the only thing that really separates a tumor promoter from a carcinogen is the dose rate at which it was applied. I just took a quick screen out of the NCI data base, the CCRIS. In there they list 94 substances classified as tumor promoters in any system, i.e., skin or any two-stage model system. Of those there have been 21 that have been assayed in a chronic type regimen, and 14 are tumorigenic in a chronic regimen. If a substance is intrinsically carcinogenic, it seems to me that it is irrelevant whether it can act in a two-stage promotion system.

DR. JAMES SWENBERG, CHEMICAL INDUSTRY INSTITUTE OF TOXICOLOGY: I'm not sure if I really caught everything you said, Ray, but getting back to Marshall's premise here, I think there probably is a good reason to try to distinguish between the cytotoxic carcinogens

and the others. I would guess, sitting here listening to all three of these talks this afternoon, that if we took some pieces out of yours, and some pieces out of Dick Kociba's and some pieces out of Marshall Anderson's, maybe we could put together a reasonable working method here.

You had 73 compounds, of which about half of the carcinogens were nonmutagenic. And Dick Kociba showed a slide where two-thirds of the carcinogens were only positive at the high dose, quoting from Joe Hase-man. It would be very interesting to see if those two line up with a much closer alignment. I would guess that they do. I think that Marshall's idea of identifying promoters by comparison of patterns of activated oncogenes between spontaneous tumors and chemically induced tumors is a potential way to do that.

I'd like to come back to something that Marshall raised about replication and promutagenic damage, and bring it back to the theme of this conference or at least what I understood was the theme of the conference. How do we get basic research findings into the risk assessment process? We really haven't dealt with that yet at this conference. Or, at least, I haven't seen very much that has dealt with it. There's been a great deal of lip service paid to this issue over the last several years, but we really don't have any methods yet that are really doing that job. Maybe we don't have the right data yet. But it would seem to me that one of the issues that we need to start addressing here is how and when are we going to start putting mechanistic data into the risk assessment process?

I maintain that one could put in the dose response for cell proliferation, and one could put in what happens with promutagenic adducts. We need to start developing those models so that this can be done. That's not going to answer the question of promoters that you've just raised here, but I think we can start addressing the quantitative aspects of risk assessment if we start incorporating some of the biology in the basic research. So I guess I'd like to turn this around and ask you and the rest of the audience how we're going to start getting this data into the process.

DR. ROBERT DEDRICK, NIH: Well, I think the answer is quite simple. Up until this afternoon's session, a very large proportion of the carcinogen assessment group from the EPA was here to listen to the presentations. And I think it gets incorporated when it gets sufficiently persuasive to get incorporated.

DR. GEORGE LUCIER, NIEHS: I'd like to raise one other issue regarding initiation promotion. I want to use an example of estrogen carcinogenicity in the Syrian hamster model. When you give diethylstilbestrol to a Syrian hamster, it gets kidney tumors. When you give estradiol, which is structurally divergent (remember John McLachlan's talk) from DES, this also gives the same high incidence of kidney tumors.

Kurt Randerath looked at the DNA adducts in the kidney after chronic exposure to these compounds and found that in both cases DNA adducts were detected by the postlabeling procedure. The interesting thing

was that the adducts were the same in both cases, which means that they weren't arising from the estrogens. They were arising from estrogen-mediated influences on either dietary constituents or endogenous factors resulting in the formation of them. But, nevertheless, these might be promutagenic lesions. They might be involved in the carcinogenic process.

So in this case would you call those estrogens genotoxic or not genotoxic? To put it in your words, it doesn't make any difference to me whether I'm walking around with adducts that arose from a hormone or a dietary constituent than exposure to the chemical. So how would you classify that chemical as an initiator or a promoter?

DR. ANDERSON: Until you know more about the structure of the adducts I'm not sure you can answer that question.

DR. LUCIER: You're absolutely right. I don't think you can evaluate the role of the adduct in the carcinogenic process until you know what the structure is and whether it's on a hot spot and so forth. But I think it was clear that because of the structural divergence that you're really not dealing with adduct formation from the estrogens themselves. So it raises a question of how do you call that? I mean, it's a carcinogen and the adducts may be involved. You have evidence that the adducts might be involved even though they, themselves, wouldn't be formed from those structures. That's somewhat of a dilemma. And I think it's appropriate for this kind of discussion if we're talking about classifying carcinogens according to stages. So it could be that if TCDD did the same thing in your model for the oncogenes that it'd be producing indirect adduction, then you may not get the same genetic lesions in the activated oncogenes as seen in the spontaneously occurring tumors. So it's an additional complexity, and I don't know how to deal with it if one is going to use initiation-promotion in the risk assessment process.

DR. ANDERSON: You would surely have to call the chemical genotoxic if it really is forming the adduct itself, or indirectly. I think the point Jim was making was valid. I think he's been trying to do it, and we have too. The product of adducts times cell replication is surely a much better dose term to use in low dose extrapolation of carcinogenic data. As far as low dose extrapolation of the carcinogenic data in rodents for genotoxic chemicals, that's obviously the way to do it. I think we've clearly shown that. Another question that I was raising is should society regulate promutagenic lesions themselves and not the carcinogenic data? I believe that this is a valid consideration.

UNKNOWN SPEAKER: Marshall, I just wanted to make a quick comment. I agree with everything you said about those mutations at the 13th and the 117th with the proviso, and I think you would agree too, that we have to learn more about the potential genotoxicity aside from the negative Salmonella.

The other thing I didn't hear a comment on was the original dilemma you posed about formaldehyde. I don't recall the first slide, but I thought the formaldehyde

data indicated a less than linear response for DNA lesions as the dose decreases. And maybe that might give you a little comfort to appreciate Jim's view that with some chemicals you do have increased efficiency at low dose. I don't know if that will help you have some degree of comfort if it turns out that the number of adducts are somewhat less than linear.

DR. SWENBERG: Let me just address that, because I don't think it came across real clearly from the slides. What you have in that slide was covalent bindings divided by exposure parts per million. So that it has a linear phase at the low end and a linear phase at the high end and a nonlinear portion in between there. And I think Marshall didn't get it quite right. Because the point was that it's linear at the high end. At 6 and at 15 ppm you have linear covalent binding, but you have a very nonlinear tumor response. And the only explanation that I can come up with is cell proliferation. I think it's a very reasonable one from a science standpoint. There's another point that needs to be made on your promutagenic adduct issue. We all must remember that all adducts are not created equal. They don't have equal potency, they don't have equal half-times, and they differ in different cell types and different tissues. The examples range from O<sup>6</sup>-methylguanine, which ends up being persistent in brain at about 10% at the highest dose for 6 months. The cisplatin that you referred to. We have other adducts. We could take O<sup>6</sup>-methylguanine in the liver, and it's virtually all repaired. It's first order, so there's always going to be a little something left over. We're not going to get rid of it all. And we have to bring in the efficiency for causing mispairing and the time that these adducts hang around in the tissues that they're causing the tumors in. It's a far more complex thing. And then you get into site-specific mutagenesis. So as I said, we've got a tremendous way to go before we're ever there completely. But I think we already have enough data that we can start. And that's what I would encourage.

DR. ROY ALBERT, UNIVERSITY OF CINCINNATI MEDICAL CENTER: I think, fundamentally, as I said at the beginning of the discussion the first day, it is anticonstructive to continue to try to view the process of carcinogenesis in an initiation-promotion framework. I think we should not only not regulate on that basis; we shouldn't even necessarily even think on that basis. And TCDD is both the best and worst of all examples because it is one of the most potent carcinogens that we've seen. It not only promotes spontaneously occurring tumors, but it induces uncommon tumors. TCDD's lesions in DNA obviously occur, because Bill Greenlee presented results here that showed that you alter the differentiation pattern of human keratinocytes. We know that it's a potent inducer of enzymes. It causes essentially irreversible enzyme activation or deregulation. And there's a known sequence 5' to the *Ah* locus in human cells that is specifically responsive to a TCDD ligand complex. It obviously is genotoxic. It changes phenotypes heritably, but it's not mutagenic. It certainly is

a promoter because it acts in a two-stage system, but it's carcinogenic when you administer it in a chronic regimen.

I think TCDD is an example of why the two-stage conception is not universally valid. That's why you can get an activated *ras* out of TCDD potentially that won't involve either a 61st or a 118th codon mutation. It may well nonmutagenically activate the *ras* gene simply through the growth factor linkage. You're heritably altering phenotypes.

DR. ANDERSON: Can Julian have a shot?

DR. JULIAN PETO, ROYAL CANCER HOSPITAL, ENGLAND: I said this yesterday, and it's been said at every risk assessment meeting I've been to for a thousand years. Is anybody even prepared to defend the use of the term "promoter"? I know everybody keeps standing up and saying that it's ridiculous. But I mean is anybody here—are there representatives of EPA here? Who's prepared to defend its use in risk assessment? Because it obviously is completely contradictory and silly. I mean, everybody who has spoken as a scientist on risk assessment has pointed it out at every level.

DR. ANDERSON: But the point is that it surely has meaning mechanistically. Cells that have alterations like activated *ras* or *neu* or other oncogenes respond differently than normal cells to agents like TCDD, phenobarbital, etc., that will selectively give growth advantage either in a negative sense or a positive sense to this cell that already has a change in it. From that sense it makes sense to differentiate between the two. I think Ray's example of TCDD is really not good. Because I think we're all walking around, not just the B5 mouse, with initiated cells sitting there waiting for something to be done. I think your data shows that with the radiation. Now whether you want to regulate it, whether you want to call it a carcinogen, I don't know. But mechanistically it makes sense. And I think the oncogene story drives it home if you look at the data in totality.

DR. PETO: But the point is—it's obviously right, I mean the stuff that Henry Pitot showed and the stuff that you're showing in the epidemiology. It's quite clear that you're interacting with things that are going on anyway spontaneously. Therefore, anything that acts at any stage is a complete carcinogen. I mean, the word "complete carcinogen" doesn't have any meaning.

DR. ANDERSON: I didn't use that term.

DR. PETO: That's the point. That's why for risk assessment purposes to make the distinction is silly and dangerous. I've shown radiation as a promoter. So it doesn't mean that you don't have to worry about it as much as if it was an initiator.

DR. ANDERSON: Why have you shown it's a promoter?

DR. PETO: Quite clearly it's a late stage action.

DR. ANDERSON: But it might be active genotoxically in that stage.

DR. PETO: Doesn't "promoter" mean a nongenotoxic carcinogen? What is the definition of a promoter? I don't know what the definition is.

DR. ANDERSON: Well, to me it's an agent that selectively causes a clonal expansion of a certain cell type.

DR. PETO: Okay, but that's a totally different meaning. Is that what's happening there? Is that what radiation is doing?

DR. ALBERT: I think that the excitement about promoters in carcinogenesis from the risk assessment standpoint is that those agents that interact through cell receptor mechanisms give the promise of being able to define low-level dose response, which from a mechanistic standpoint in a way which contrast between a single molecule, single hit, process that we're essentially locked into.

The other comment I'd like to make is that although the interaction between adduct levels and cell proliferation makes a beautiful story, I think there ought to be some reservations about the extent to which it's applicable to different systems. We've just got some pilot data. Admittedly, it's pilot data. But the chronic application of benzo[a]pyrene to the mouse skin up through the time of tumor formation, which begins at about 7 months, so that the exposures are quite substantial, doesn't produce any detectable change in the cell turnover rate.

Now, to be sure, the skin has an inherent turnover rate, but it raises the question as to whether there is necessarily a quantitative relationship between cell proliferation and adducts that can explain the time and magnitude of tumor response. It may be yes, but I've found that result to be kind of a bucket of cold water.

DR. ANDERSON: But you couldn't induce tumors with just one single dose at that low-dose level. You had to give it repeatedly. So the probability of having a cell undergoing replication greatly increases.

DR. SWENBERG: I didn't get it all down because I couldn't write fast enough, but Henry Pitot gave us the

definition of a promoter that was accepted at the recent promotion meeting. It had two aspects to it: reversible expansion of initiated cells and/or reversible alteration of genetic expression. And Julian said we didn't have a definition of it. That was the definition that was given. I didn't hear anybody counter it at the time. It's a reasonable definition that we can start working from.

DR. ANDERSON: That's a good place to end, Jim.

DR. SWENBERG: One last comment for Roy. You know, this business of being locked into one hit, one molecule, there is no evidence to support that. No evidence at all. It's all dogma.

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